spatially discordant alternans. Spatially discordant alternans can also arise from electromechanically concordant alternans and electromechanically discordant alternans in different regions of the tissue. In conclusion, fibroblast-myocyte coupling has multiple pro-arrhythmic effects on electrophysiological properties in cardiac tissue.

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Gap Junction Permeability: Transfer of Negative and Positive Charged Probes

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Gap junction channels are composed of connexins, which exhibit specific permeability to the variety of larger solutes including second messengers, polypeptides and siRNAs.

Here, we report the permeability of solutes with different size and net charge Lucifer Yellow (443 (no counterion), -2); Carboxyfluorescein (376, -2); AlexaFluor350 (326, -1); Ethidium bromide (314, +1) and NBD-m-TMA (280, +1) through gap junction channels in HeLa cells expressing Cx26, Cx40, Cx43 and Cx45.

The channel permeability was determined using simultaneous measurements of junctional conductance and the cell-cell flux of a fluorescent probe.

All four connexins transferred negative charged probes: LY, CF and AF350, however, Cx40 and Cx26 exhibited reduced permeability when compared to Cx43. These connexins revealed following permeability ratios for LY/ CF/ AF350, respectively, relative to the ubiquitous cation K+: 0.029/ 0.032/ 0.069 for Cx43; 0.014/ 0.021/ 0.049 for Cx45; 0.0044/ 0.014/ 0.0245 for Cx26 and 0.0026/ 0.0034/ 0.0206 for Cx40.

The positive charged NBD and EthBr exhibited the following permeability relative to K+: 0.045 and 0.0125 for Cx43; 0.048 and 0.0036 for Cx45; 0.055 and 0.026 for Cx26; 0.040 and 0.009 for Cx40.

In summary, all negative charged species showed a similar permeability order: Cx43>Cx45>Cx26>Cx40. For positively charged species the permeability orders were: Cx26≈Cx43≈Cx40≈Cx45 (NBD) and Cx26≥Cx43≈Cx40>Cx45 (EthBr). Reduced EthBr permeation through Cx45 channels in comparison to other connexins suggests a size-dependent discrimination of the solute. However, the reduced correlation between junctional conductance and positive charged probes flux suggests intracellular binding of the solute. Therefore quantitative comparison of positively charged solutes has to be taken cautiously.

These results confirm that channels formed from individual connexins can discriminate for solutes based on size and charge suggesting that channel selectivity may be a key factor in cell signaling.

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1459-Pos Board B303

Stem Cell Transplantation Induces a Rapid Change in Cardiac Excitability

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Embryonic (ES) and bone marrow derived stem cells are discussed as a potential source for cardiac replacement tissue. Transplantation of undifferentiated cells into the cardiac infarct region has been shown to decrease infarct size and preserve cardiac function. Long term studies determined homing of stem cells in the cardiac muscle, the immediate impact of cell transplantation on the electrophysiological properties of however remains unclear. To determine if the time course in which stem cells establish intercellular coupling we plated Calcein/AM loaded ES cells on monolayers of cardiomyocytes (HL-1 cells). Dye transfer that was monitored by confocal microscopy, was first observed 60 min after heterocellular culture was established. It could be blocked by carbenoxolone indicating the presence of gap junction channels. After 200 min 36 ± 7% of ES cells had established intercellular coupling with cardiomyocytes. To determine the impact of cell transplantation on the electrophysiological properties we established monolayers on multielectrode arrays (MEAs). From field potential measurements we determined that induction of co-culture resulted in a biphasic change of the electrophysiological properties. During the first 45 min an increase of the conduction velocity (CV: 142 ± 17 %) and of the spontaneous beating frequency (F: 172 ± 11%) could be detected (n = 10). With further progression of co-culture however, a continuous decrease of excitability occurred (180min; F: 31 ± 3%; CV: 50 ± 2.5%) that ultimately resulted in the loss of spontaneous activity (210 min). In control cultures no biphasic change in F or CV was observed. The data indicate that stem cell transplantation results in rapid heterocellular coupling between stem cells and cardiomyocyte and a suppression of cardiac excitability. The contribution of intercellular coupling and other paracrine mechanism to the change in excitability remains to be determined.

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Posttranslational Modifications Of Connexin26 Identified By MALDI-TOF/TOF Mass Spectrometry

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Gap junctions play important roles in auditory function. Mutations in the Cx26 gene are the predominant cause of inherited nonsyndromic deafness. Some Cx26 deafness mutations directly disrupt the intercellular molecular and/or ionic signaling pathway, while others may affect the location and character of posttranslational modifications (PTMs) governing channel assembly, function and biological regulation. Mass spectrometry was used to determine if Cx26 PTMs occur at sites of deafness-causing mutations. Cx26 was isolated from HeLa cells at >95% purity by immunoaffinity followed by metal chelate chromatography using carboxyl-terminal hexahistidine and hemagglutinin tags. In-gel and in-solution enzymatic digestions were carried out in parallel with trypsin, chymotrypsin and endoproteinase-GluC. Peptides were recovered with and fractionated from reverse-phased C8 beads by stepwise elution with increasing concentrations of organic solvent. Using an ABI4800 MALDI-TOF/TOF-MS, spectra were acquired from each elution step, thereby improving detection of low abundance peptides in complex mixtures and maximizing sequence coverage. Acquisition, processing and interpretation parameters were further optimized to improve ionization and fragmentation of hydrophobic connexin peptides. MALDI-TOF-MS and MALDI-TOF-MS/MS sequence coverage values obtained were significantly above those reported for other mammalian membrane proteins. Total Cx26 sequence coverage by MALDI-TOF-MS was 75.2%, with 31.1% sequence confirmed by MALDI-TOF-MS/MS. Improved ionization and sequencing of Cx26 peptides (especially transmembrane pore-lining domains) were further achieved with a Waters nano-liquid chromatography-coupled electrospray ionization quadrupole-TOF-MS. Several different PTMs of Cx26 were identified, many of which were at sites of deafness-causing mutation. The PTMs included phosphorylation, acetylation, methylation, citrullination, hydroxylation, γ-carboxylation and palmitoylation. Knowledge of the location and character of Cx26 PTMs will be instrumental in guiding experiments to understand how cellular mechanisms of channel regulation can become altered and lead to losses in auditory function. Supported by GM36044, DC7470, NS56509 (ALH) & NS046593 (HL).

1461-Pos Board B305

Mutagenesis Of Charged Residues In The N-terminal α-helix Of Connexin37 Reveals An Essential Lysine Residue

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Connexins are transmembrane protein subunits that combine to form cell surface hexameric hemichannels and intercellular gap junction channels. They contain a short cytoplasmic N-terminus that has been implicated in channel gating and oligomerization. Using NMR, we previously showed that the N-terminus of human connexin37 (CX37) is α-helical between amino acids 5 and 16. The α-helical region has a hydrophilic face with three aligned negatively charged residues (E8, D12 and E16) and one positively charged residue (K9). To test their function, these charged amino acid residues were mutated individually to alanines or to other neutral amino acids. The CX37 mutants were tested for formation of gap junction plaques by fluorescence microscopy after transient transfection of HeLa cells and for formation of functional hemichannels by two-microelectrode voltage clamp after expression in single *Xenopus* oocytes. Each of the negatively charged amino acid alanine substitution (E8A, D12A, or E16A) or charge neutralization (E8Q, D12N, or E16Q) mutants formed gap junction plaques. These mutants all formed conducting hemichannels; ionic currents were of comparable magnitude to those in oocytes expressing wild-type CX37 when measured without divalent cations and were blocked by 2 mM external calcium. While gap junction plaques were observed in HeLa cells transfected with K9A, this construct did not form conducting hemichannels in *Xenopus* oocytes. No plaques were detected in a charge reversal mutant at this position (K9E). These results suggest that the negatively charged residues within the N-terminal α-helix are not individually required for formation of functional channels. In contrast, the positively charged residue, K9, is required for hemichannel opening and influences formation of gap junction plaques.

1462-Pos Board B306

Extracellular ATP Mediates The Intercellular Ca2+ Wave Induced By Mechanical Stimulation In Human Salivary Gland Cells

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Intracellular Ca2+ signaling has a central role in regulation of salivary gland cell function. Coordination of Ca2+ signaling between cells contributes to synchronized and effective secretion of saliva. However, mechanisms that underlie this signaling remain elusive. Here, intercellular Ca2+ waves (ICW) and their propagation in human salivary gland (HSG) cells were investigated using fura-2 fluorescence imaging. While not well understood, mechanical stimulation of a single cell in a cluster with a micropipette induces ICW. The Ca2+ signal is propagated from the stimulated cell to the 7-9th tier of cells or ~120 μm. The following findings indicate that ICW propagation in HSG cells uses an extracellular and ATP-dependent pathway. The purinergic receptor antagonist suramin significantly decreased ICW propagation. Extracellular ATP or UTP abolished ICW suggestive of receptor desensitization. Gap junction intercellular communication is not involved in ICW in HSG cells because the gap junction inhibitor oleamide did not inhibit ICW. Furthermore, HSG cells showed poor dye coupling upon microinjection of Lucifer Yellow. The Ca2+ transients observed within each cell are dependent on Ca2+ release from the ER as thapsigargin abolished the ICW. The phospholipase C inhibitor U73122 also blocks ICW indicating that these transients are IP3-dependent. Furthermore, store operated Ca2+ entry (SOCE) modulates the amplitude of Ca2+ signal since removal of extracellular Ca2+ or a SOCE inhibitor SK&F 96365 decreased the amplitude of Ca2+ signal. Inhibition of mitochondrial Ca2+ uptake with FCCP/oligomycin or ruthenium red showed similar effects on the amplitude. These results indicate that propagation of this ICW utilizes extracellular ATP, likely through the P2U(P2Y2) receptor in HSG cells. The major Ca2+ mobilization mechanisms are IP3-dependent ER Ca2+ release and SOCE. Finally, mitochondrial energy metabolism and Ca2+ uptake modulated this ICW propagation.

Emerging Single Molecule Techniques II

1463-Pos Board B307

Distortion of Protein Receptor Decreases the Lifetime of Receptor-ligand Bond

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Ligand competition assay is often used in single-molecule force spectroscopy (SMFS) to test the specificity of binding. We have noticed that in the SMFS measurements that utilize biotin tethered to the tip of an atomic force microscope and streptavidin bound to the surface, addition of ~1mM of free biotin in solution does not completely eliminate binding events as detected by SMFS. We hypothesize that the compressive force applied to the streptavidin-biotin complex on the substrate during the measurements shortens the bond lifetime. We have tested this hypothesis by performing a series of measurements with different maximum compressive force applied to the surface. These measurements indicate that the compressive force affects the number of interactions measured in the presence of free biotin. The measured dependence agrees with the model that takes into account the increase of the tip-surface contact area with an increase of the maximum applied force. These results indicate that for SMFS to be used as a competition assays, shortening of a lifetime of the receptor-ligand bond by compressive force should be considered.

1464-Pos Board B308

Immobilization of Single Biomolecules Using Covalent-Bond Linkages for Fluorescence Single-Molecule Experiments

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The streptavidin-biotin bridge is commonly used in single-molecule studies to surface immobilize biomolecules onto microscope slides. However, the presence of tryptophanes impedes utilization of UV light and numerous fluorescent nucleotide analogs, such as 2-aminopurine. We are developing new approaches to immobilize DNA/RNA molecules without use of streptavidin and biotin. One approach consists of using the Huisgen cycloaddition reaction between an alkyne and an azide, which is an example of ''click'' chemistry reaction. In this ''click'' chemistry approach, 3′-azide modified oligos are immobilized to an alkyne-modified microscope slide surface through a triazole linkage. This cycloaddition reaction is very stable in many physiologically relevant buffers, and has been shown to occur without the need of a catalyst. In another approach, we take advantage of the efficient coupling between thiol groups to immobilize biomolecules by forming disulfide bridges. 3′-thiol modified oligos are surface immobilized on a thiol-modified microscope slide by forming disulfide bonds. We are currently improving the immobilization efficiency by optimizing the reaction parameters and conditions. We anticipate that these approaches will allow us to investigate local conformational changes in biomolecular systems at the single molecule level.

1465-Pos Board B309

A Flexible Anti-Brownian Electrokinetic (ABEL) Trap for Single-Molecule Immobilization in Solution

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We demonstrate our ability to trap and probe individual fluorescent particles in solution using an improved anti-Brownian electrokinetic (ABEL) trap. Traditional single-molecule immobilization techniques include surface attachment and laser tweezers; the former technique often disrupts fragile biochemical systems, while the latter requires that molecules be conjugated to large beads. The ABEL trap circumvents these issues by tracking the motion of a particle via fluorescence, and applying electrokinetic feedback forces to cancel its Brownian motion.

Our ABEL trap suppresses the Brownian motion of a fluorescent particle as follows. A laser beam is rapidly steered in a small scan pattern near the center of a microfluidic cell. An avalanche photodiode detects fluorescence photons from the molecule. A field-programmable gate array compares the precise arrival time of each photon with the known position of the laser, and generates a corresponding feedback voltage. The feedback voltages is amplified and applied to the trap. We use a broadband supercontinuum laser with an acousto-optic tunable filter to enable fluorescent tracking in any part of the visible spectrum, and we scan the laser using electro-optic deflectors that can function at up to 100 kHz. This combination of hardware enables precise spatial, temporal, and spectral control of our illumination and detection optics and can apply feedback at a latency of 2 μs, a better-than-tenfold improvement over previous trap designs. We hope that these improvements will enable us to trap single small-molecule fluorophores in solution.

The flexibility of the ABEL trap makes it amenable for a wide variety of biophysical studies. Work is currently underway to apply the ABEL trap to study the dynamics of DNA in solution. In the future, we hope to apply the trap to study the kinetics of proteins such as proteorhodopsin.

1466-Pos Board B310

Automating Optical Tweezers Experiments With a Microfluidic Laminar Flow Channel Device

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Optical tweezers perform in singulo experiments on biological reactions that occur stochastically, often through multiple pathways. Characterization of single molecule trajectories allows determination of conformational distributions and detection of intermediates. However, this approach requires repeating the measurements tens or hundreds of times to achieve sufficient statistics. Aiming for high-throughput experiments, we combine microfluidic delivery of beads into the assay chamber with automated optical tweezers.

We have developed a computer controlled microfluidic device for nano-litre sample-handling. Feedback control is achieved by monitoring and setting the pressure differences between individual inlet reservoirs and the outlet with high (<1 Pa, ca. 0.1 mmH2O) precision. This allowed us to achieve stable, repeatable, fluid flow in the micron-sized channels of a typical lab-on-chip setup. As a proof-of-principle experiment we performed repeated force-extension measurements on ~10 kb dsDNA-molecules. Preliminary results on automatic assembly of the dumb-bell assay (bead-DNA-bead construct) and force-extension measurements will be presented. These automated, high-throughput, single-molecule experiments allow us to study rare events and phenomena in nanoscale biological physics, often inaccessible to other methods.

1467-Pos Board B311

Experimental Apparatus for Simultaneous Trapping And Nanometer-precision Localization of Single Biomolecules

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The development and continuous improvement of single molecule techniques have elucidated the mechanics of numerous ubiquitous subcellular processes step-by-step, previously inaccessible by conventional average-based biochemical studies. At present, particularly fruitful is the combination of different single molecule techniques in the same setup. In this work we have developed an experimental apparatus which allows the simultaneous detection of the position of a single processive biomolecule, together with mechanical control of its